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RNA polymerase II/III transcription specificity determined by TATA box orientation

(*in vitro* transcription/basal transcription machinery/TATA box-binding protein/transcription factor IID/transcription factor IIIB)

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ABSTRACT **The TATA box sequence in eukaryotes is located about 25 bp upstream of many genes transcribed by RNA polymerase II (Pol II) and some genes transcribed by RNA polymerase III (Pol III). The TATA box is recognized in a sequence-specific manner by the TATA box-binding protein (TBP), an essential factor involved in the initiation of transcription by all three eukaryotic RNA polymerases. We have investigated the recognition of the TATA box by the Pol II and Pol III basal transcription machinery and its role in establishing the RNA polymerase specificity of the promoter. Artificial templates were constructed that contained a canonical TATA box as the sole promoter element but differed in the orientation of the 8-bp TATA box sequence. As expected, Pol II initiated transcription in unfractionated nuclear extracts downstream of the "forward" TATA box. In distinct contrast, transcription that initiated downstream of the "reverse" TATA box was carried out specifically by Pol III. Importantly, this effect was observed regardless of the source of the DNA either upstream or downstream of the TATA sequence. These findings suggest that TBP may bind in opposite orientations on Pol II and Pol III promoters and that opposite, yet homologous, surfaces of TBP may be utilized by the Pol II and Pol III basal machinery for the initiation of transcription.**

The TATA box was originally identified as a regulatory signal upstream of many protein-coding genes transcribed by RNA polymerase II (Pol II). However, some tRNA and 5S RNA genes and most RNA polymerase III (Pol III)-transcribed genes with external promoters also possess TATA boxes ≈25–30 bp upstream of the transcription start site. When present in Pol III promoters, the TATA box can have a significant effect on the efficiency and accuracy of transcription of these genes by Pol III (1–6). Interestingly, a better match to a canonical TATA sequence often becomes apparent if the TATA sequences in Pol III promoters are read in the opposite orientation. As one example, transcription of plant small nuclear RNA (snRNA) genes by either Pol II (U1, U2, U4, and U5) or Pol III (U3 and U6) requires a TATA box (7). However, the 8-nt consensus sequence for plant Pol II-transcribed genes is 5′-TATAAAAN-3′ (a canonical TATA sequence), whereas the consensus sequence for plant Pol III-transcribed snRNA genes is 5′-TTTATATA-3′ (7). (The complementary strand sequence, in this case 5′-TATATAAA-3′, represents the better match to a canonical TATA sequence.) Other examples of genes with TATA sequences that can be better interpreted as inverted TATA boxes range evolutionarily from the human U6 and 7SK genes to several yeast tRNA genes (5, 8–10).

These and other observations prompted us to investigate whether the orientation of the TATA box, in the absence of any additional promoter elements, could be a primary determinant of RNA polymerase specificity. Indeed, by utilizing a strong canonical TATA box sequence and a nuclear extract with similar amounts of Pol II and Pol III activity, we find that a "forward" TATA box specifically directs Pol II transcription, whereas a "reverse" TATA box specifically directs Pol III transcription.

MATERIAL AND METHODS

Construction of Templates. The D "Forward" TATA and D "Reverse" TATA templates (shown in Fig. 1) were constructed in two steps. First, synthetic DNA sequences that represent a hybrid of the *Drosophila* U1 and U6 snRNA gene transcription start sites (11, 12) were inserted between the *Bam*HI and *Xba* I restriction sites of the polylinker of pUC18. After isolation of this recombinant plasmid, synthetic oligonucleotides that contained the TATA sequence in either the forward or reverse orientation were inserted between the *Kpn* I and *Bam*HI restriction sites of the polylinker.

To construct the H/D "Forward" TATA and H/D "Reverse" TATA templates (shown in Fig. 2), synthetic oligonucleotides containing a combination of DNA sequences near the transcription start sites of human U1, U2, and U6 snRNA genes (8, 13, 14) were inserted between the *Eco*RI and *Kpn* I restriction sites of the D "Forward" TATA and D "Reverse" TATA templates. This placed the human sequences in the opposite orientation and in an upstream position relative to the *Drosophila* sequences.

To construct the pUC18 "Forward" TATA and pUC18 "Reverse" TATA templates (shown in Fig. 3), the *Drosophila*-like sequences were deleted from the D "Forward" TATA and D "Reverse" TATA templates by digestion with *Bam*HI and *Sal* I. After filling in the overhanging ends with the Klenow fragment of DNA polymerase, the plasmids were recircularized. Plasmids were grown in *Escherichia coli* TOP10 cells (Invitrogen), purified by using Qiagen (Chatsworth, CA) Plasmid Midi kits, and used as templates for *in vitro* transcription.

***In Vitro* Transcription Assays.** Transcription reactions (25 μl final volume) were carried out for 1 h at 25°C with 10 μl of soluble nuclear fraction (SNF) prepared from *Drosophila* embryos (15–17), 5.5 μl HEMG buffer [25 mM Hepes, pH 7.6/12.5 mM $MgCl_2$/0.1 mM EDTA/10% (vol/vol) glycerol/1.5 mM dithiothreitol] containing 0.1 M KCl, 7.5 μl of ribonucleoside triphosphate (rNTP) mix (1.7 mM each rNTP in 67 mM Hepes, pH 7.6), and 2 μl of plasmid DNA (0.2 mg/ml in 10 mM Tris·HCl, pH 8.0/1 mM EDTA). The drugs α-amanitin (a specific inhibitor of Pol II but not insect Pol III) and tagetitoxin [Tagetin, Epicentre Technologies, Madison, WI; a specific Pol III inhibitor (18)] were added to the indicated reactions at final concentrations of 2 or 200 μg/ml and 400 units/ml, respectively.

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Abbreviations: Pol II, RNA polymerase II; Pol III, RNA polymerase III; TBP, TATA box-binding protein; SNF, soluble nuclear fraction; TFIID, transcription factor IID; TFIIIB, transcription factor IIIB; snRNA, small nuclear RNA.

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Purification of transcription products and analysis by primer extension were as described (16). The ^{32}P-labeled primers [New England Biolabs catalog number 1233 or a primer (1211z) similar to New England Biolabs catalog number 1211 but longer by 7 nt at its 5′ end] were complementary to pUC18 DNA on opposite sides of the polylinker. Primer-extension products were separated by electrophoresis through 10% denaturing polyacrylamide gels and subjected to autoradiography at −70°C. The transcription initiation sites were mapped at high resolution by running the primer-extension products on similar gels together with sequencing ladders generated by using the same ^{32}P-labeled primers (data not shown).

RESULTS

The relevant portions of the two constructs used as templates for the initial experiment are shown at the top of Fig. 1. These two plasmids, D "Forward" TATA and D "Reverse" TATA, are identical to each other except for the polarity of the 8 bp that make up the TATA box (boldface type). An 8-bp sequence was chosen because the TATA box-binding protein (TBP) contacts exactly 8 bp in its cocrystal structure with DNA (21, 22). Also, 5′-TATAAAAA-3′ represents a canonical TATA sequence that, on the basis of footprinting assays, is a high-affinity binding site for TBP (unpublished data). Moreover, previous studies indicated that this sequence is a strong promoter element that can function independently of other promoter elements for the initiation of transcription (16). Finally, this TATA sequence is highly asymmetrical—i.e., dissimilar in sequence—in its 5′ and 3′ halves. Conceivably, these properties should maximize the ability of TBP, as a component of either transcription factor IID (TFIID) or transcription factor IIIB (TFIIIB), to recognize the TATA box with high specificity and directionality.

The synthetic sequences inserted to the right of the TATA boxes contain no known promoter sequences but were designed to bear some resemblance to the *Drosophila* U1 and U6 snRNA gene transcription start sites (11, 12) to potentially optimize the context for initiation of transcription by either Pol II or Pol III. However, these constructs contain no proximal sequence element which is essential for snRNA gene expression and is normally located ≈40–65 bp upstream of the transcription start site of snRNA genes. The plasmid templates were transcribed *in vitro* by using SNF prepared from *Drosophila* embryos (15, 17).

Identical sets of reactions were carried out, except that one set contained the D "Forward" TATA template (Fig. 1, lanes 1–5) and the complementary set contained the D "Reverse"

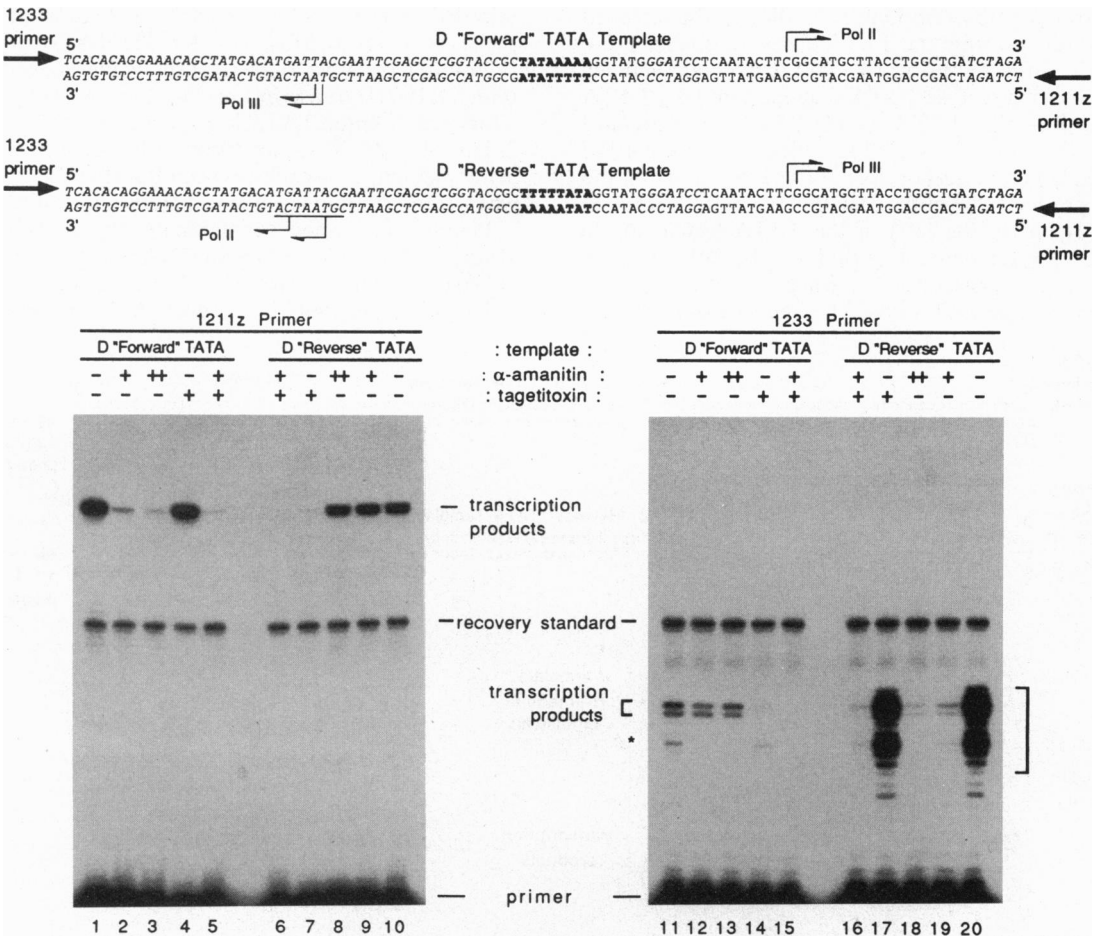

FIG. 1. Transcription *in vitro* of D "Forward" TATA and D "Reverse" TATA templates using a *Drosophila* SNF. The plasmid templates are identical in sequence except for the orientation of the 8-bp TATA box (boldface type). The D indicates that synthetic DNA resembling a combination of sequences near the *Drosophila* U1 and U6 snRNA gene transcription start sites was placed to the right of the TATA boxes. Plasmid (pUC18) DNA sequence is shown in italics. Transcription products were analyzed by primer extension, and the specific start sites are indicated by arrows above or below the DNA sequence. Bands are indicated on the autoradiograms that correspond to reverse transcription products of 76 or 77 nucleotides (1211z primer, lanes 1–10) and 38–45 nucleotides (1233 primer, lanes 11–20). An asterisk indicates a minor Pol II transcription product detected by the 1233 primer arising from the D "Forward" TATA template. The position of a 54-mer oligonucleotide that was added to each reaction as a recovery standard is also shown. Plus signs above the individual lanes indicate inclusion of the Pol III-specific inhibitor tagetitoxin (400 units/ml) or the Pol II-specific inhibitor α-amanitin at either a low concentration (+, 2 μg/ml) or a high concentration (++, 200 μg/ml). Note that insect Pol III is resistant to both low and high concentrations of α-amanitin (19, 20).

TATA template (lanes 6–10). Transcription was monitored by primer extension from an oligonucleotide primer (1211z) complementary to pUC18 DNA downstream of the synthetic *Drosophila* DNA. With no inhibitors present, transcription products were readily detected from both templates (Fig. 1, lanes 1 and 10). Mapping the start sites at high resolution (data not shown) indicated that the transcription products from both templates initiated at identical positions 22 or 23 nucleotides downstream of either the forward or the reverse TATA box.

To distinguish which RNA polymerases were responsible for the synthesis of the observed transcription products, the Pol II-specific inhibitor α-amanitin and the Pol III-specific inhibitor tagetitoxin (18) were employed. Transcription from the forward TATA template was due to Pol II since it was inhibited by α-amanitin (Fig. 1, lanes 2 and 3) but was resistant to tagetitoxin (Fig. 1, lane 4). In stark contrast, transcription arising from the reverse TATA template was due to Pol III because it was inhibited by tagetitoxin (Fig. 1, lane 7) but was resistant to inhibition by α-amanitin (Fig. 1, lanes 8 and 9). [Note that insect Pol III is completely resistant to high as well as low concentrations of α-amanitin (19, 20).]

In the next experiment (Fig. 1, lanes 11–20), transcription reactions were carried out under exactly the same conditions as used for lanes 1–10 except that the 1233 primer was instead used to monitor the products. By using the 1233 primer, it is possible to assay for transcription proceeding in the leftward or upstream direction from the TATA boxes. In this scenario, the TATA box in the D "Forward" TATA template actually takes on the role of a reverse TATA sequence, and the TATA box in the D "Reverse" TATA template becomes a forward TATA sequence. Lanes 11–14 of Fig. 1 demonstrate that Pol III was the polymerase responsible for the synthesis of the transcription products that initiated within the pUC18 DNA 22 or 23 nt upstream (to the left) of the TATA box in the D "Forward" TATA template. In other words, Pol III again specifically initiated transcription downstream of a sequence equivalent to a reverse TATA box. The intensity of transcription is lower than that observed in lanes 8–10 (Fig. 1), probably due to the absence of a highly compatible Pol III initiation site in the plasmid DNA. Conversely, the D "Reverse" TATA template, when assayed with the 1233 primer (Fig. 1, lanes 16–20), yielded a cluster of α-amanitin-sensitive products that initiated in the plasmid DNA 20–27 nt to the left of the TATA box. Thus, as expected, the canonical TATA box orientation specifically selected Pol II.

To circumvent the heterogeneity associated with transcription initiation in the pUC18 DNA, a composite sequence resembling DNA near the beginning of the human U1, U2, and U6 snRNA genes was inserted to the left of the TATA boxes of the previous templates. The new constructs were designated H/D "Forward" TATA and H/D "Reverse" TATA. The relevant DNA in these constructs is shown at the top of Fig. 2. When transcription of these templates was assayed by using the 1211z primer, the results were essentially identical to those obtained in Fig. 1 (compare Fig. 1, lanes 1–10, with Fig. 2, lanes 1–10). Thus, inclusion of the human DNA to the left of the forward and reverse TATA boxes had no detectable effect on the formation of the respective Pol II and Pol III transcription complexes oriented in the direction of the *Drosophila* DNA.

When transcription was assayed in the direction of the human DNA by using the 1233 primer, products from both the forward and reverse TATA templates were observed that initiated sharply at a CG dinucleotide 23 or 24 nt to the left of the TATA boxes. Most notably, the TATA box in reverse orientation (relative to the start site being assayed) specifically directed Pol III transcription (Fig. 2, lanes 11–15). In contrast, a forward-oriented TATA box specifically selected Pol II (Fig. 2, lanes 16–20). Thus, inversion of the TATA box resulted in a complementary switch between Pol II and Pol III specificity when transcription was assayed from either direction.

Finally, we wished to eliminate any possibility that the synthetic *Drosophila* or human DNA sequences in the previous constructs could be responsible for the determination of RNA polymerase specificity in the preceding experiments. For this

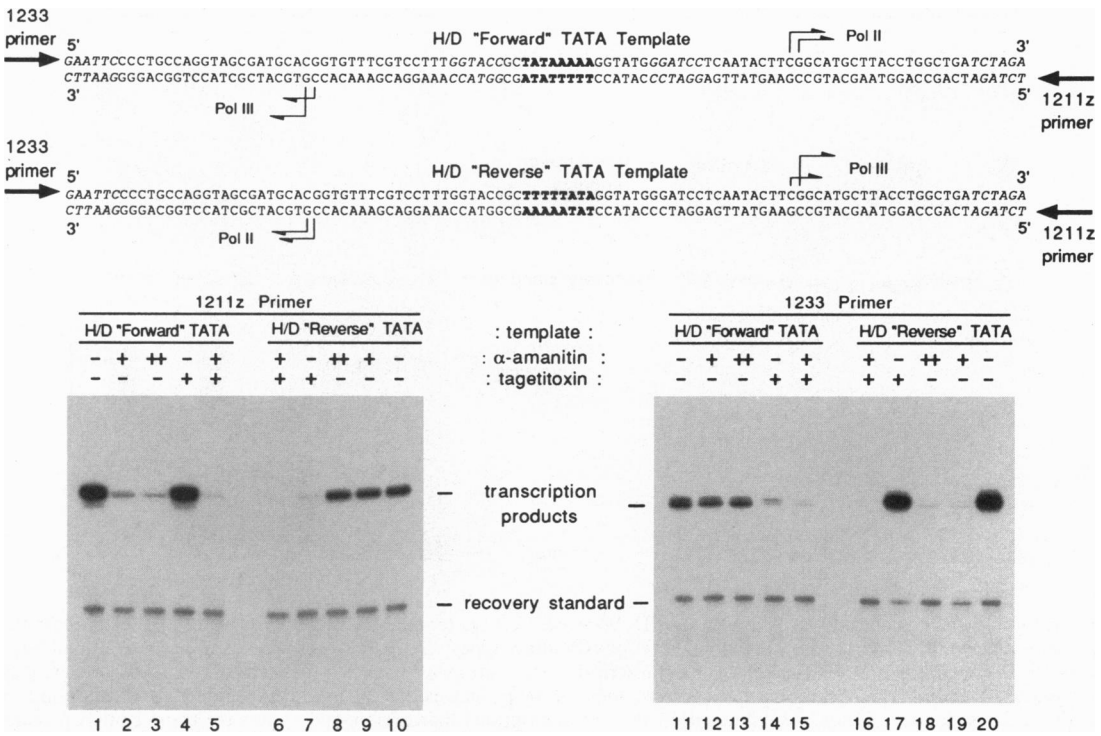

FIG. 2. Transcription *in vitro* of H/D "Forward" TATA and H/D "Reverse" TATA templates. The plasmid templates were similar to those illustrated in Fig. 1 except that synthetic DNA resembling a combination of sequences near the human U1, U2, and U6 snRNA gene transcription start sites was inserted to the left of the TATA boxes. Primer extension of transcription products yielded bands of 76 or 77 nt (1211z primer) or 71 or 72 nt (1233 primer).

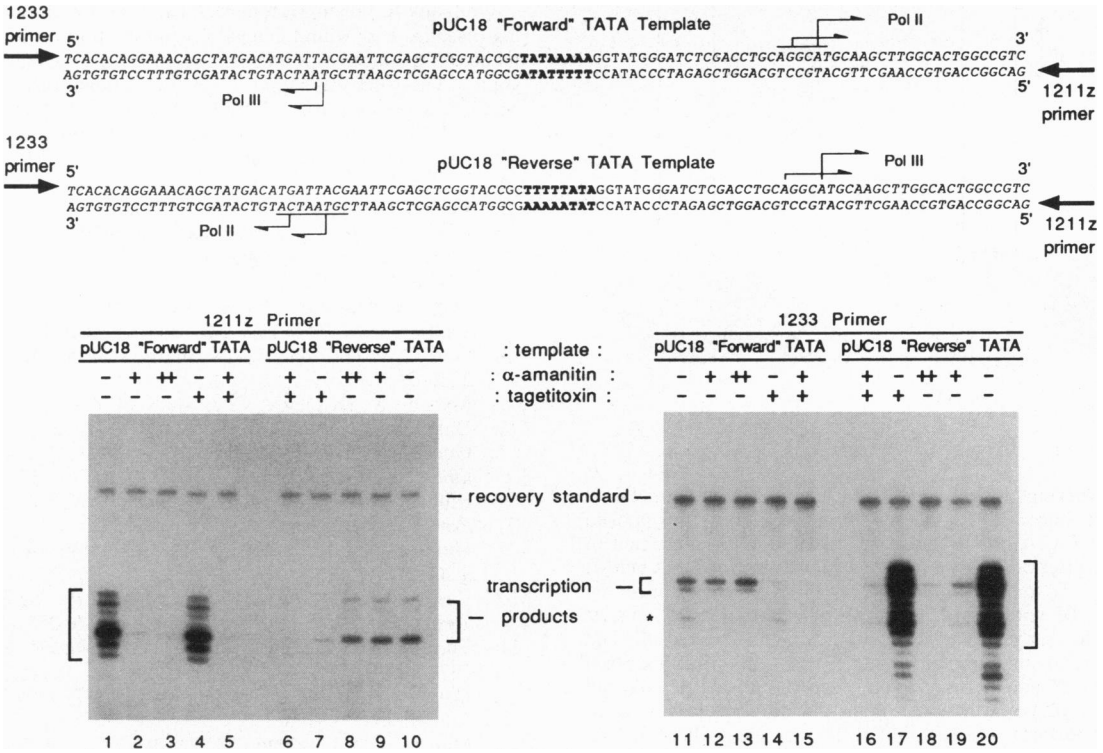

FIG. 3. Transcription *in vitro* of pUC18 "Forward" TATA and pUC18 "Reverse" TATA templates. The plasmid templates contained no eukaryotic DNA other than the TATA box sequences cloned in each direction. Primer extension of transcription products yielded bands of 36–42 nt (1211z primer) or 38–45 nt (1233 primer).

purpose, a final pair of constructs was tested in which all of the synthetic DNA resembling eukaryotic sequences was deleted except for the forward and reverse TATA boxes (Fig. 3). As might be expected, transcription originating in both directions within the plasmid DNA exhibited start-site heterogeneity, but the polymerase specificity followed precisely the pattern previously observed—i.e., forward TATA, Pol II; reverse TATA, Pol III. Thus, the polarity of the TATA box is able to determine Pol II/Pol III specificity in the absence of any additional eukaryotic DNA.

DISCUSSION

In the experiments described above, the TATA box was the only eukaryotic promoter element present in the plasmid constructs. The forward TATA box specifically promoted Pol II transcription, and the reverse TATA box specifically promoted Pol III transcription. The selection of polymerase was independent of all combinations of upstream and downstream sequences examined. In effect, when transcription in both directions is taken into account, four different upstream sequences and four different sequences downstream of the TATA box were present in the three constructs tested.

Although TATA-mediated Pol II transcription is taken for granted, TATA-mediated Pol III transcription in the absence of other promoter elements has not been as thoroughly examined. Nevertheless, highly purified TFIIIB, Pol III, and a TATA box are known to be sufficient for transcription *in vitro* at the yeast U6 snRNA gene promoter (23–25). TATA-dependent transcription has also been reported in vertebrate systems in the context of natural mRNA promoters (26–29), but in these less-purified vertebrate systems, a mixture of both Pol II and Pol III transcription heading in the same direction was normally observed. Moreover, high levels of TATA-mediated Pol III transcription *in vitro* were generally dependent upon the use of fractionated mammalian extracts or preincubation of the template with the TFIIIB-containing phosphocellulose fraction B (26, 28, 29).

Our experiments have employed an unfractionated transcription extract containing both Pol II and Pol III activity (the highly active *Drosophila* SNF). The only variable in these experiments was the polarity of the TATA box within otherwise identical templates. The asymmetry of the chosen TATA box (5′-TATAAAAA-3′), together with its high affinity for TBP, may contribute to the high degree of Pol II/Pol III selectivity exhibited by this system. Our findings linking TATA box orientation and polymerase specificity are supported by the work of Benfield and coworkers (28, 29) who, using a fractionated mammalian system, observed that Pol III transcription was favored by a reverse TATA box.

Our results also demonstrate that divergent transcription by Pol II and Pol III from the TATA box can take place within the same reaction mixture. However, we should not assume that Pol II and Pol III can simultaneously transcribe the same DNA template molecule, albeit in opposite directions. Since the template is in excess in the *in vitro* reaction mixtures (15), TFIID and TFIIIB (which both contain TBP) may associate with different subpopulations of the identical DNA molecules in a reaction mixture. Thus, it is quite possible that any individual DNA molecule may be transcribed only by Pol II or only by Pol III.

At first thought, it is a rather startling observation that the orientation of the 8-bp TATA box can determine RNA polymerase specificity. What is the mechanistic linkage between TATA box orientation and polymerase choice? Although a firm conclusion cannot be reached from the present data, it is reasonable to discuss potential mechanisms as a basis for further research. First, it is conceivable that different TBP-associated factors in TFIID and TFIIIB subtly alter the DNA-binding specificity of TBP, such that TBP in TFIID may preferentially recognize a forward TATA box, whereas TBP in TFIIIB may preferentially bind a reverse TATA box. However, a more straight-forward interpretation of our results is illus-

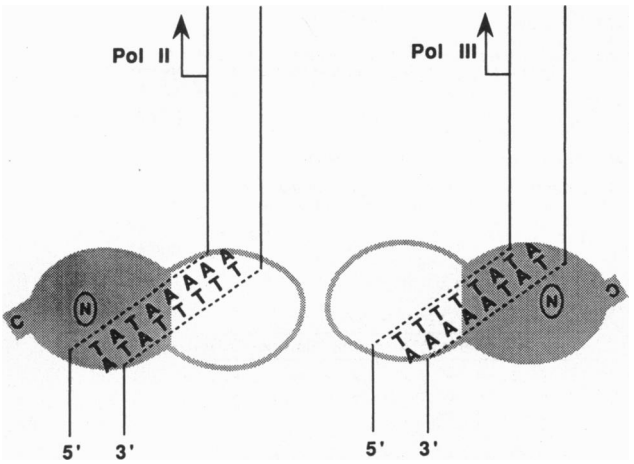

FIG. 4. A working model for pol II/III selection by the directional binding of TBP. Since TBP contacts exactly 8 bp of DNA, the opposite polarity of the TATA box (left and right diagrams) is presumed to direct different faces of TBP (viewed from above with the DNA below) toward the Pol II and Pol III transcription start sites. Since the orientation of TBP with respect to Pol II transcription initiation sites is inferred from cocrystal structures (21, 22), the shaded ellipsoid represents the TBP domain that contains the amino and carboxyl termini (labeled N and C respectively), and the unshaded ellipsoid represents the evolutionarily homologous domain that has an essentially identical tertiary structure but differs by more than 60% in primary structure (30). The model suggests that the Pol II and Pol III basal transcription machineries may interact with homologous, but opposite, surfaces of TBP.

trated in Fig. 4. This model deserves consideration because it is consistent with structural data from x-ray crystallography (21, 22, 30), it has interesting evolutionary implications, and it represents a function for TBP in polymerase selection. In the diagram presented in Fig. 4, the TBP–DNA interaction is considered to be essentially identical whether TBP is a component of TFIID or TFIIIB, but the reversal of the TATA box directs opposite faces of TBP toward the transcription start site. Since TBP has two domains (and thus two faces) that are homologous yet subtly different in structure, perhaps one face of TBP has coevolved primarily with components of the Pol II basal transcription machinery—e.g., TFIIB and Pol II—and the other face of TBP has coevolved primarily with components of the Pol III basal transcription machinery—e.g., the TFIIB-related factor BRF (31) and Pol III. Proof that different faces of TBP are directed toward Pol II and Pol III transcription start sites will likely require detailed protein interaction studies and the determination of additional cocrystal structures.

At most natural promoters, it is quite possible that the orientation of the TATA box may play a role subordinate to that of other nearby promoter elements. This should be particularly true at promoters that lack canonical or asymmetrical TATA boxes. Factors bound to other proximal and distal cis-acting elements may normally play the primary role in recruiting TFIID or TFIIIB to the promoter (with TBP in the proper orientation) to ensure both correct polymerase specificity and correct direction of transcription. For example, a strong transcription factor IIIC (TFIIIC)-binding site (composed of properly spaced A and B boxes) can be a dominant element in the determination of Pol III specificity due to TFIIIC-mediated recruitment of TFIIIB in the proper orientation for Pol III transcription (25, 32, 33). Similarly, a Pol II initiator element, recognized by the TBP-associated factor $TAF_{II}150$ (34–36), would favor the recruitment of TFIID, with TBP in the proper orientation for Pol II transcription.

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